Tissue Microenvironment Modulates CXCR4 Expression and Tumor Metastasis in Neuroblastoma[1]

Libo Zhang[*,†], *Herman Yeger*[†], *Bikul Das*[*,†], *Meredith S. Irwin*[‡,§] *and Sylvain Baruchel*[*,‡,§]

*New Agent and Innovative Therapy Program, The Hospital for Sick Children, Toronto, Canada; [†]Department of Pediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, Canada; [‡]Division of Hematology and Oncology, Department of Pediatrics, The Hospital for Sick Children, Toronto, Canada; [§]Institute of Medical Sciences, University of Toronto, Toronto, Canada

Abstract

Neuroblastoma (NB) is derived from intrinsic migratory neural crest cells and has a high potential for distant metastasis. Growing evidence has implicated chemokine receptors, especially CXCR4, which normally control immune and inflammatory cell migration, as having important roles in tumor progression. In this study, we investigated the expression of CXCR4 in eight different NB cell lines and found that CXCR4 expression is dynamically regulated in NB and can be modulated by different tissue stromata. In addition, we demonstrate that IL-5 and IFN-γ are released from stromal cells and act as differential mediators for CXCR4 expression. We also overexpressed CXCR4 in two NB cell lines, NUB-7 and SK-N-BE(2), and studied the role of CXCR4 in NB metastasis both *in vitro* and *in vivo*. *In vitro* transwell invasion assay showed that CXCR4 overexpression promoted NB cell migration preferentially toward a bone marrow stromal cell–conditioned medium. Using an *in vivo* xenograft model, CXCR4-overexpressing cells showed an increased incidence of metastasis, most notably bone marrow metastasis. Our studies reveal critical roles for CXCR4 in NB metastasis and provide insights into the regulatory mechanism of chemokine receptors in NB and the importance of the tissue microenvironment in modulating tumor cell behavior.

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Introduction

Chemokines represent a large superfamily of small peptides that currently comprise 42 members in humans, which recruit different cell populations by interacting with seven transmembrane domain G-protein–coupled receptors. At present, 18 chemokine receptors have been identified. The primary role of chemokines/receptors is to regulate the recruitment and trafficking of leukocyte subsets to inflammatory sites through chemoattraction by activating leukocyte integrins that bind to their adhesion receptors on endothelial cells [1,2]. Chemokines are also involved in neuronal cell migration and patterning [3]. Recent studies suggest that many cancers express an extensive network of chemokines and chemokine receptors [4,5]. These tumors are characterized by dysregulated production of chemokines and abnormal chemokine receptor expression. It has become evident that chemokines are able to couple to distinct signaling pathways. Most chemokines share the ability to activate G-protein–sensitive phospholipase C isoforms, resulting in inositol-3,4,5-trisphosphate generation. Some of the chemokines can also inhibit adenylate cyclase, activate MEK-1 and ERK-1/2, and stimulate the tyrosine phosphorylation of focal adhesion complex components [6–8].

Among chemokine receptors, CXCR4 is frequently studied because it is expressed by most cancer types, including cancers of epithelial, mesenchymal, and hematopoietic origin [9]. For example, tumor cells from breast, prostate, pancreatic, lung, and ovarian carcinomas, as well as glioblastomas, all express CXCR4 [10–15]. CXCL12 is the only known ligand for CXCR4. It is found at sites of metastasis in breast and thyroid cancers [16]. Considering their original role in the trafficking of hematopoietic stem cells to the bone marrow [17], CXCR4 and CXCL12 (also known as SDF-1) are believed to be involved in tumor growth and metastasis to the bone marrow.

Neuroblastoma (NB) is derived from embryonic neural crest cells that form the peripheral sympathetic nervous system and have a high potential to migrate. Metastatic NB has a high mortality rate; thus, understanding the mechanism of how NB tumor cells invade and metastasize will help in designing more effective therapies to control the development and metastasis of NB, and will further help to develop an animal model that more faithfully resembles tumor metastasis in patients. So far,

Address all correspondence to: Sylvain Baruchel, New Agent and Innovative Therapy Program, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. E-mail: sylvain.baruchel@sickkids.ca

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most studies on NB chemokine system have been performed *in vitro* with the SK-N-SH cell line and its subclone SK-SY5Y. Using flow cytometry, Geminder et al. [18] demonstrated the expression of CXCR4 in eight different NB cell lines (SH-SY5Y, CHP126, NHB, LAI55N, KELLY, SK-N-MC, NBL-WN, and SK-N-SH). A recent report demonstrated that a higher expression of CXCR4 was found in primary NB from patients with high-stage disease and in patients with bone and bone marrow metastases. Clinical outcome in patients with tumors expressing high levels of CXCR4 is significantly worse than in patients with low CXCR4 tumor expression [19]. All these studies support the hypothesis that specific chemokines/receptors may play important roles in NB tumor cell behavior, but more direct evidence is required to establish the role of chemokines/receptors in NB cell invasion and metastasis. In this study, we screened chemokine/receptor profiles in eight different NB cell lines and investigated the roles of CXCR4 in NB tumor growth and progression using mouse xenograft models. We also demonstrate the key role of stromal cells in NB metastasis and a potential regulatory mechanism for CXCR4 in NB.

Materials and Methods

Cells and Antibodies

Eight human NB cell lines were used in this study. SK-N-BE(2), IMR-32, and SK-N-SH cells were obtained from ATCC (Manassas, VA). LAN-5 cells were kindly provided by Dr. Robert Seeger (Children's Hospital Los Angeles, Los Angeles, CA), NBL-S cells were from Dr. Susan Cohn (Children's Memorial Hospital, Chicago, IL), and SK-N-BE(1) cells were from Dr. R. A. Ross (Fordham University, Bronx, NY). The GOTO NB cell line was purchased from Riken Gene Bank (Riken, Tsukuba, Ibaraki, Japan). All NB cell lines were cultured at 37°C in 5% CO_2 in α-MEM (Multicell; Wisent, Inc., St. Bruno, Quebec, Canada) with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco, Carlsbad, CA). Antibodies to CXCR4 were purchased from Abcam (Cambridge, MA) (ab2090) and R&D Systems (Minneapolis, MN; clone 44708). The CXCR4 inhibitor AMD3100 was obtained from Sigma (St. Louis, MO). CXCR4–pBABE was kindly provided by Dr. Nathaniel Landau (The Salk Institute for Biological Studies, La Jolla, CA). HFK293TV cells and retroviral plasmid 10A1 were generously provided by Dr. H. Vaziri (Ontario Cancer Institute, Ontario, Canada).

Primary Culture of Stromal Cells

Stromal cells were established as primary cultures from different organs of NOD/SCID mice, including the lung, liver, bone, bone marrow, and adrenal gland. Briefly, after sacrificing the mice, organs were dissected, and fat and connective tissues were removed. Tissues were minced with scissors into 1-mm cubes and digested in 15 ml of phosphate-buffered saline (PBS) containing 75 mg of collagenase type II at 37°C, with rapid shaking. After digestion, cells were washed once with 10 ml of α-MEM supplemented with 10% FBS. The cells were plated and allowed to adhere to flasks overnight. The medium was changed on the following day and thereafter at 3- to 5-day intervals. The cultures were continuously observed for growth of cells with mesenchymal morphology and were passaged thrice. Bone marrow stromal cells were isolated from bone marrow aspirates from the femurs of NOD/SCID mice. To obtain conditioned media, confluent stromal cells were cultured in α-MEM with 10% FBS for 48 hours, and the conditioned medium was harvested, filtered, and used for the induction of tumor cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

The measurement of SDF-1α was carried out using ELISA kit (R&D Systems) according to the manufacturer's instructions. Units of fluorescence activity were converted to actual concentrations by a standard curve.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was prepared by Qiagen RNeasy mini kit (Qiagen, Valencia, CA). Residual DNA was eliminated using the Qiagen RNase-Free DNase Set (Qiagen; cat. no. 79254). Reverse transcription was carried out as described by the manufacturer. DNA was amplified with the specific primers listed in Table 1. Resulting PCR products were subjected to electrophoresis in 2% agarose gels, and resulting bands were recorded under UV light to identify product specificity and reaction sensitivity.

Immunoblots

Aliquots of total protein extracts (50 μg) were run on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunodetection involved specific primary antibodies, appropriate secondary antibodies conjugated to horseradish peroxidase, and ECL plus chemiluminescence detection system (Amersham Biosciences). Equal loading and transfer were monitored by probing for α-tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Western blot analyses were quantified using the ImageJ 1.32 software (National

Table 1. Primers Used By RT-PCR.

CXCR4	
Forward	5′-TCATCTACACAGTCAACCTCTACA-3′
Reverse	5′-GAACACAACCACCCACAAGTCATT-3′
SDF-1	
Forward	5′-CTTCAGACACTGAGGCTCCC-3′
Reverse	5′-AGGCAATCACAAACCCAGTC-3′
CCL2	
Forward	5′-CAAACTGAAGCTCGCACTCTCGCC-3′
Reverse	5′-ATTCTTGGGTTGTGGAGTGAGTGTTCA-3′
CCL3	
Forward	5′-GCTGACTACTTTGAGACGAGC-3′
Reverse	5′-CCAGTCCATAGAAGAGGTAGC-3′
CCR7	
Forward	5′-TCCTTCTCATCAGCAAGCTGT-3′
Reverse	5′-GAGGCAGCCCAGGTCCTTGAAG-3′
GAPDH	
Forward	5′-TGATGACATCAAGAAGGTGGTGAAG-3′
Reverse	5′-TCCTTGGAGGCCATGTGGGCCAT-3′

Institutes of Health, Bethesda, MD) after densitometric scanning of films.

Immunocytochemistry

NUB-7 cells were seeded on coverslips and incubated for 24 hours at 37°C. The coverslips with cells were then fixed with 4% paraformaldehyde in PBS for 10 minutes, washed with PBS, permeabilized in 1% Triton X-100 in PBS for 5 minutes, washed, and blocked with PBS with 1% bovine serum albumin (BSA). For CXCR4 staining, cells were incubated with mouse anti-human CXCR4 monoclonal antibody diluted in 1% BSA–PBS (0.01 mg/ml) for 1 hour at room temperature, rinsed thrice with PBS, and then incubated for 30 minutes with secondary antibody (cy3-conjugated goat anti-mouse IgG; Molecular Probes, Eugene, OR) diluted in 1% BSA–PBS (1:100). After washing, coverslips were mounted and examined under a Zeiss Axioskop fluorescence microscope (Zeiss, Standort Göttingen, Germany).

Overexpression of CXCR4

For CXCR4 overexpression, HFK293TV cells were infected with the combination of pBABE–CXCR4 and retroviral plasmid 10A1 using SAINT-MIX (Synvolux Therapeutics, Amsterdam, The Netherlands), as described by the manufacturer. Empty pBABE vector was also used in HAFK293TV cell infection as control. The medium was changed after 48 hours, and the culture supernatant containing the CXCR4 virus was collected on days 3 and 4. The supernatant was filtered through a 0.45-μm Millex HV filter (Millipore, Billerica, MA), mixed with 8 μg/ml polybrene, and added directly to NB cell culture. Stable transfectants of CXCR4 were established by puromycin (1.0 μg/ml) screening. CXCR4 expression was assessed by RT-PCR and Western blot analysis. Polyclonal populations form both CXCR4 transfectants were used for further *in vitro* and *in vivo* experiments.

Cell Proliferation Assay

Cell proliferation was measured with Alamar Blue assay according to manufacturer's protocol (Trek Diagnostics Systems, Inc., Cleveland, OH). Briefly, Alamar Blue was diluted 1:10 in the cell culture medium, and color change was monitored after 3 hours. Colorimetric evaluation of cell proliferation was performed using a SPECTRAmax Gemini spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA), with 540 nm as excitation wavelength and 590 nm as emission wavelength, and values were expressed as relative fluorescence units.

Cell Adhesion Assay

Briefly, 24-well plates (Costar; Corning, Acton, MA) were coated with rat tail collagen at 37°C for 1 hour, with some wells left uncoated as negative controls. After washing the plates with PBS, blocking buffer (0.5% BSA in DMEM) was added at 37°C for 1 hour. A total of 1×10^5 cells/well was plated in 250 μl of α-MEM plus 1 mg/ml BSA in a 24-well plate and incubated in 5% CO_2 at 37°C for 0.5 hour. The plate was then washed thrice with PBS. The percentage of adherent cells was determined by Alamar Blue assay. Three independent experiments were carried out in triplicate.

In Vitro *Cell Invasion Assays*

Cell invasion was assessed with the BD BioCoat (BD Biosciences, San Jose, CA) tumor invasion system based on the Boyden chamber principle using 8-μm pore positron emission tomography membrane inserts coated with a layer of BD Matrigel Matrix (BD Biosciences). After detaching cells with nonenzymatic cell dissociation solution (Sigma), NB cells were incubated in suspension in α-MEM with 10% FBS for 2 hours and then seeded onto the extracellular matrix layer, which had been previously rehydrated at room temperature for 2 hours. For chemokine-dependent invasion assay, stromal cell–conditioned medium (SCM) was added to the lower chamber as chemoattractant. Cells were incubated for 48 hours at 37 °C in an incubator (5% CO_2). Invaded cells on the bottom of the insert membrane were fixed with 70% ethanol and stained with hematoxylin. For quantification, the average number of migrating cells per field was assessed by counting 10 random fields under a microscope (original magnification, ×250).

Cytokine Antibody Array

Conditioned media for the liver, lung, and adrenal gland were obtained as described above. α-MEM supplemented with 10% FBS was used as negative control. Cytokine antibody array was performed with ChemiArray system (Mouse Cytokine Antibody Array I; Chemicon International, Temecula, CA) according to the manufacturer's protocol. Briefly, array membranes were blocked with 1× blocking buffer at room temperature for 30 minutes and then incubated with conditioned media at room temperature for 2 hours. After washing thrice with 2 ml of wash buffer at room temperature, biotin-conjugated anti-cytokine primary antibody was added to each membrane and incubated at room temperature for 2 hours. Subsequently, membranes were washed thrice and incubated with diluted HRP-conjugated streptavidin at room temperature for 2 hours. Cytokines were detected by chemoluminescence reaction with exposure to Kodak X-mat film (Estman Kodak Company, Rochester, NY) at room temperature for 1 minute. Relative expression levels among different samples were compared to positive controls.

Mouse Xenograft Model

Both CXCR4 transfectants and control NUB-7 cells were injected intravenously into NOD/SCID mice at 1×10^6 cells/mouse. Mice were sacrificed after 3 or 4 weeks. For subcutaneous growth, 1×10^6 CXCR4–pBABE–transfected SK-N-BE(2) cells were injected subcutaneously, and mice were sacrificed after 3 weeks. Studies of tumorigenicity, growth rate, patterns of metastasis, tumor histology, immunocytochemistry, and molecular characterizations were conducted.

Statistical Analysis

Data represent the mean ± standard error of the indicated number of independent experiments. Differences between groups were assessed using a two-way analysis of

variance, followed by two-tailed Student's *t* test. $P < .05$ was considered significant.

Results

Expression of Chemokine and Chemokine Receptors in NB

We chose three chemokines (CCL2, CCL3, and SDF-1) and two chemokine receptors (CXCR4 and CCR7) and performed RT-PCR to demonstrate their expression profiles in eight NB cell lines and one primary culture from a multirelapse NB patient bone marrow metastasis sample, designated NB12. As can been seen by RT-PCR (Figure 1*A*), the various NB cell lines showed variable chemokine/receptor expression profiles. CCL-2 mRNA was detected only in the GOTO and SK-N-BE(2) cell lines. We also identified CCR7 and CCL3 mRNA from the multirelapsed NB sample NB12, whereas it was absent from in all NB cell lines. CXCR4 mRNA was detected in both primary tumor sample NB12 and most NB cell lines, except for GOTO. After identifying CXCR4 mRNA in those cell lines, we performed Western blot analysis and confirmed CXCR4 protein expression in five NB cell lines (Figure 1*B*).

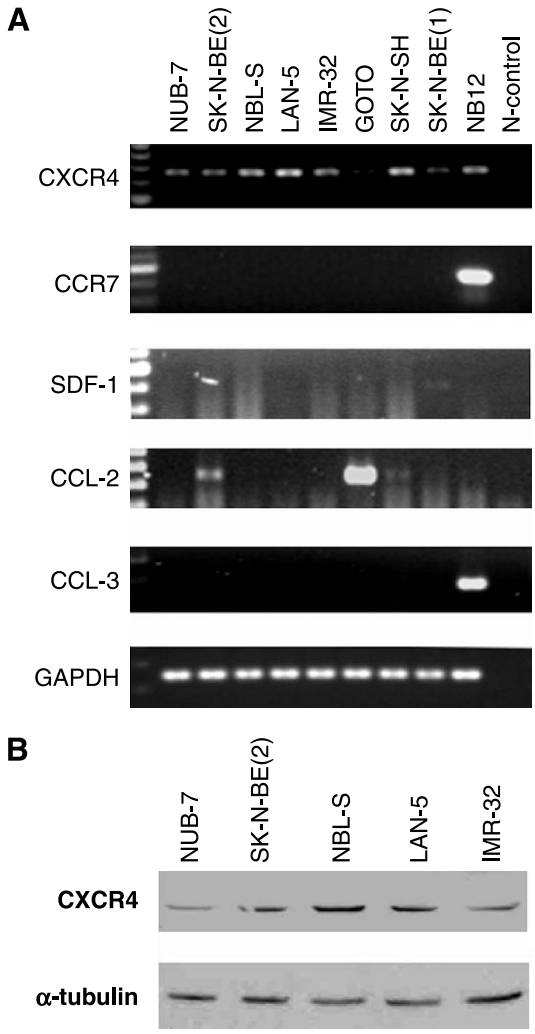


Figure 1. *Expression of chemokines and chemokine receptors in NB cell lines. (A) RT-PCR showing different profiles of the chemokine/receptor system in eight NB cell lines [NUB-7, SK-N-BE(2), NBL-S, LAN-5, IMR-32, GOTO, SK-N-SH, and SK-N-BE(1)] and one primary NB tumor sample (NB12). GAPDH was used as internal control. (B) CXCR4 expression shown by Western blot analysis in five NB cell lines [NUB-7, SK-N-BE(2), NBL-S, LAN-5, and IMR-32] relative to the α-tubulin loading control.*

Modulation of CXCR4 Expression By Stromal Cells

After investigating the expression of CXCR4 in different NB cell lines, we next addressed its potential regulatory mechanism. From the specific metastatic pattern of NB, we suspected that the tumor microenvironment, including tumor stromal cells, could play an important role in regulating chemokine receptor expression. To mimic the microenvironment of *in vivo* metastasis, stromal cells were generated from the primary culture of different normal mice organs, including the liver, lung, bone, bone marrow, and adrenal gland. CXCR4 expression was investigated after culturing the NUB-7 cell line with conditioned media from different stromal cells. Interestingly, significant changes in CXCR4 expression were observed (Figure 2) in different SCM, suggesting that chemokine receptors could be modulated by different stromal microenvironments. As shown by RT-PCR (Figure 2*A*), Western blot analysis (Figure 2*B*), and immunocytometry (Figure 2*C*), we observed a downregulation of CXCR4 expression with adrenal SCM, whereas liver SCM stimulated CXCR4 expression in NUB-7 cells. Taken together with our studies in intravenous NOD-SCID mouse metastatic model using NUB-7 and demonstrating that liver metastasis presented before metastases to other sites, it is likely that modulation of chemokine receptors is one of the preliminary steps required for early NB cell migration. Regulation of CXCR4 expression by different SCM was also observed in two other NB cell lines LAN-5 and NBL-S. As shown in Figure 2*D*, for the LAN-5 cell line, all SCM could upregulate CXCR4 expression; for the NBL-S cell line, adrenal gland SCM could suppress CXCR4 expression and bone SCM could upregulate CXCR4 expression.

CXCR4 Expression Is Regulated by IL-5 and IFN-γ Produced from Stromal Cells

To gain insight into the potential molecular mechanisms underlying CXCR4 expression, we investigated the production of cytokines from different stromal cells and their roles in CXCR4 expression. Cytokine antibody array was performed using SCM from different origins, including the adrenal gland, liver, and lung. As shown in Figure 3*A*, among the 22 cytokines screened, both IL-5 and IFN-γ are released from liver, adrenal gland, and lung stromal cells. However, liver stromal cells released much less IL-5 compared to the adrenal gland and lung, and the IFN-γ/IL-5 ratio was significantly higher in liver SCM than in lung and adrenal SCM.

This high IFN-γ/IL-5 ratio released form liver SCM prompted additional experiments in which CXCR4 expression was analyzed after exposing NUB-7 to different concentrations of recombinant IL-5 and IFN-γ *in vitro*. Indeed, CXCR4 expression was upregulated with only 0.1 ng/ml

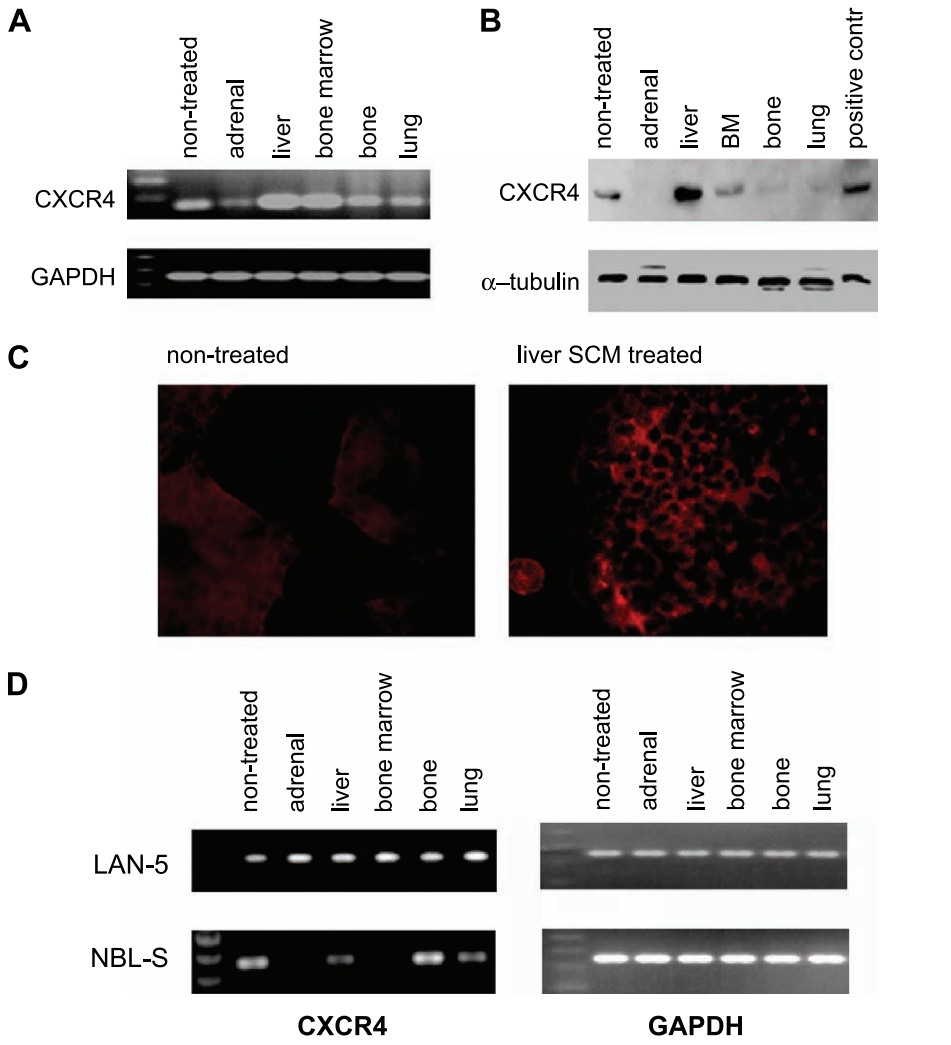


Figure 2. *Chemokine receptors induced by stromal cells. Stromal cells were isolated from different mouse organs: adrenal gland, liver, bone marrow, bone, and lung. (A) Expression of CXCR4 is affected by SCM. NUB-7 cells were incubated with conditioned media from different stromal cells for 48 hours under normal culture conditions. Expression of CXCR4 was studied by RT-PCR. (B) Western blot analysis showing the distinct effects of stromal cells on CXCR4 expression in NUB-7 cells. HeLa cell lysate was used as positive control. (C) Immunofluorescence staining of CXCR4 in liver SCM–induced NUB-7 cells and nontreated NUB-7 cells. Permeabilized CXCR4-overexpressing and control NUB-7 cells were incubated with anti-CXCR4 monoclonal antibody followed by Cy3-labeled (red) secondary antibody. Immunoreactive CXCR4 was located in the cytosol and membrane of those cells. (D) Regulation of CXCR4 expression by different SCM in two other NB cell lines, LAN-5 and NBL-S. Expression of CXCR4 was studied by RT-PCR.*

IFN-γ, and a dramatic decrease in CXCR4 expression was noted with IL-5 treatment at a concentration of 1 ng/ml (Figure 3*C*), suggesting a possible explanation for the mechanism by which liver SCM could stimulate CXCR4 expression.

Overexpression of CXCR4 in NB Cell Lines

To investigate the role of CXCR4 in NB development, we overexpressed CXCR4 in two NB cell lines NUB-7 and SK-N-BE(2). NUB-7 and SK-N-BE(2) cells are well characterized, and both have relatively low levels of CXCR4. NB cells were transfected with pBABE–CXCR4 or the empty vector pBABE–Con, and cells were selected with puromycin and cultured under these conditions for about 1 month. Both RT-PCR (Figure 4*A*) and Western blot analysis (Figure 4*B*) showed that CXCR4 levels were significantly upregulated in CXCR4-overexpressed cells compared with pBABE–Con and nontransfected cells.

Expression of CXCR4 Affects NB Cell Invasiveness

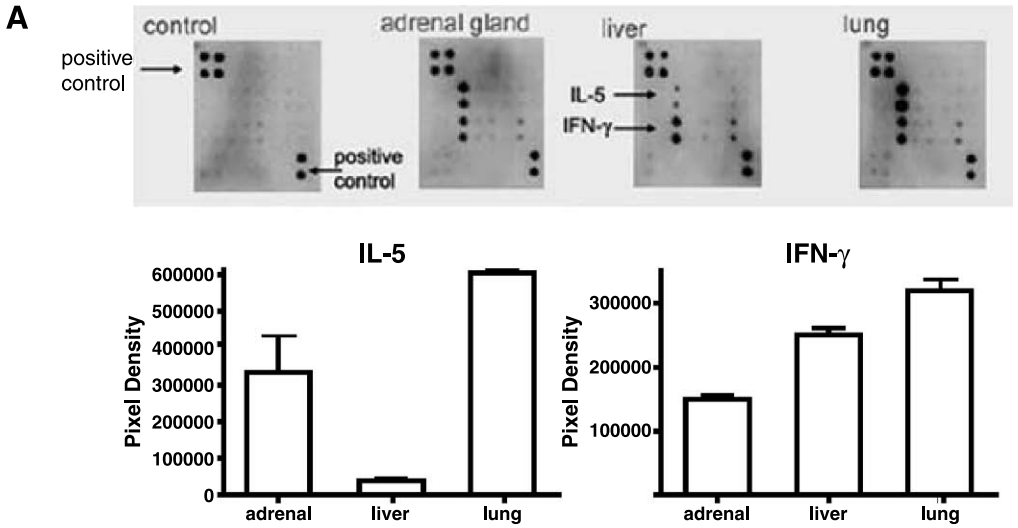
To establish the role of CXCR4 in NB behavior, we performed a proliferation assay with Alamar Blue, a cell adhesion assay using rat tail collagen, and a cell migration assay in Matrigel-coated transwell plates. We observed no significant effect on the proliferation of NUB-7 cells after CXCR4 overexpression (Figure 4*C*). Similarly, NUB-7 adhesion was not significantly affected by either overexpressing CXCR4 or blocking CXCR4–SDF-1 interaction by adding neutralizing antibodies or AMD3100 to cell culture (Figure 4, *D* and *E*).

In cell invasion assay, we used two different chemoattractants in the transwell invasion system, including liver SCM and bone marrow SCM. As shown in Figure 4*F*, after CXCR4 overexpression, a dramatic increase in cell migration toward bone marrow SCM was observed, compared with control NUB-7 cells ($P < .05$). In contrast, we observed no significant change in cell migration toward liver SCM even

after CXCR4 overexpression, suggesting preferential migration toward the bone marrow. To determine the chemoattractant driving CXCR4-mediated NB cell migration, we assessed the SDF-1 level in conditioned media from liver or bone marrow stromal cells. As shown in Figure 4*G*, using ELISA, we demonstrated a significantly higher SDF-1α level in bone marrow SCM (1206.93 ± 155.24 pg/ml) than in liver SCM (275.61 ± 115.18 pg/ml; $P < .01$).

CXCR4 Expression Regulates NB Metastasis In Vivo

To gather further support for the role of CXCR4 *in vivo*, two mouse xenograft models were used: NB cells injected subcutaneously or intravenously to NOD/SCID mice. In the subcutaneous model, both CXCR4-expressing NUB-7 cells and control cells formed localized tumors with no significant differences in tumor size (data not shown). In the intravenous model, mice were injected with 1×10^6 CXCR4-



B

ChemiArray™ Mouse Cytokine Antibody Array I Map

1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12	IL-12p70	IL-13
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12	IL-12p70	IL-13
5	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNF RI	TNF-α
6	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNF RI	TNF-α
7	TPO	VEGF	Blank	Blank	Blank	Blank	Blank	Pos
8	TPO	VEGF	Blank	Blank	Blank	Blank	Blank	Pos

Pos, positive control; Neg, negative control. All others used are standard abbreviations.

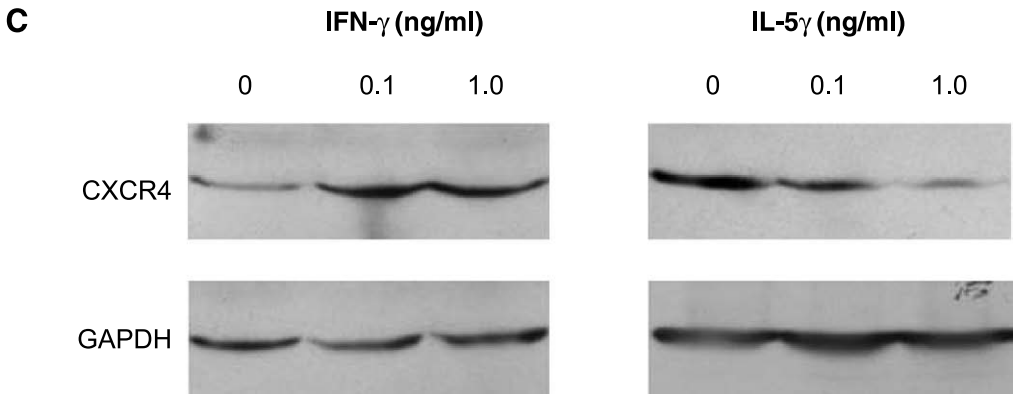


Figure 3. *(A) Cytokine antibody array with SCM from the adrenal gland, liver, and lung. Twenty-two cytokines were screened in three SCM. A conditioned medium was obtained as described above. α-MEM supplied with 10% BS was used as negative control. Array was performed with the ChemiArray system according to the manufacturer's protocol. (B) The map of mouse antibody array I can be used to identify individual cytokines. (C) CXCR4 expression is regulated by recombinant IL-5 and IFN-γ in NUB-7 cells. NUB-7 cells were cultured for 48 hours with different doses of mouse recombinant IL-5 and IFN-γ (0, 0.1, and 1 ng/ml). CXCR4 expression was assessed by Western blot analysis.*

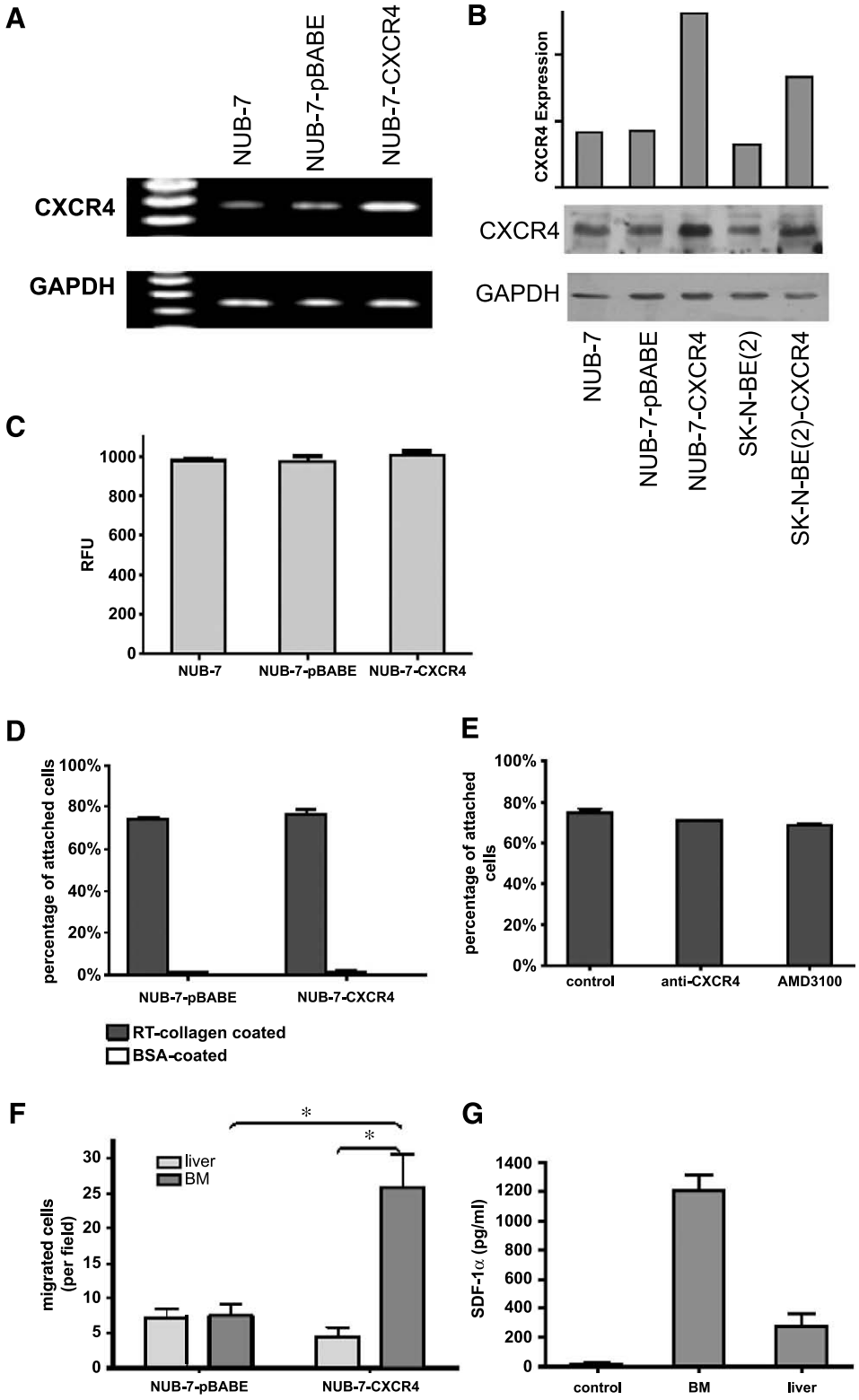


Figure 4. *Overexpression of CXCR4 in NB cell lines. For CXCR4 overexpression, both NUB-7 and SK-N-BE(2) cells were infected by CXCR4–pBABE. Empty pBABE vector was used as control. (A) RT-PCR showing the expression level of CXCR4 after CXCR4 overexpression in the NUB-7 cell line. (B) Western blot analysis confirmed the overexpression of CXCR4 in both NUB-7 and SK-N-BE(2) transfectants. Blots were scanned and analyzed using ImageJ software (The National Institutes of Health [NIH], Bethesda, MD). (C) Effect of CXCR4 overexpression on NUB-7 cell proliferation. Cell proliferation was measured with Alamar Blue assay after 4 days. All experiments were carried out in triplicate. (D and E) Effect of CXCR4 expression on NB cell adhesion. Cell adhesion assay was performed using NUB-7 cells and control cells (C). NUB-7 cells were also treated with neutralizing antibody to CXCR4 (5 μg/ml) or specific CXCR4 inhibitor AMD3100 (1 μM) for 10 minutes at normal culture conditions. Cell adhesion assay was performed as described in the Materials and Methods section. (F) Effect of CXCR4 expression on NB cell invasiveness. Transwell cell invasion assay was performed using liver or bone marrow SCM as chemoattractant. Invaded cells on the bottom of the insert membrane were fixed with 70% ethanol and stained with hematoxylin. For quantification, the average number of migrating cells per field was assessed by counting 10 random fields under a microscope (original magnification, ×250). Results are representative of three separate experiments. *P < .05, compared with each control. (G) Release of SDF-1α from stromal cells. Liver and adrenal gland SCM were obtain as described above. The measurement of SDF-1α was carried out using ELISA kits (R&D Systems), according to the manufacturer's instructions. α-MEM supplemented with 10% FBS was used as negative control.*

Table 2. Dissemination of NUB-7 in NOD/SCID Mice after Intravenous Injection of CXCR4-Overexpressing Cells.

Group	*n*	Lung [*n* (%)]	Liver [*n* (%)]	Kidney [*n* (%)]	Bone Marrow [*n* (%)]
NUB-7–pBABE	7	2 (28.6)	7 (100)	6 (85.7)	1 (14.3)
NUB-7–CXCR4	7	3 (42.9)	7 (100)	6 (85.7)	6 (85.7)

CXCR4 was overexpressed in NUB-7 cells. CXCR4-overexpressing NUB-7 cells (1×10^6; NUB-7–CXCR4) and vector control cells (NUB-7–pBABE) were injected intravenously to NOD/SCID mice. Three weeks after injection, the mice were sacrificed. Metastatic site formation was determined histologically after hematoxylin–eosin staining. For each organ site, the number of animals with disseminated NB is indicated, and the dissemination frequency is shown in parentheses.

overexpressing or control NUB-7 cells. After 3 weeks, liver, kidney, and lung metastases were observed in both groups. However, only one of seven mice in the control group developed bone marrow metastasis, whereas six of seven mice in the NUB-7–CXCR4 group had bone marrow metastasis (Table 2 and Figure 5*A*). Thus, CXCR4 overexpression enhanced NB cell migration toward the bone marrow. Strikingly, CXCR4-overexpressing SK-N-BE(2) cells and control cells also produced similar size subcutaneous tumors; however, extensive liver, kidney, and bone marrow metastases developed only in the CXCR4 overexpression group (Figure 5, *B* and *C*). All metastases were confirmed by histologic analysis (data not shown). These results suggest that the expression of CXCR4 could also stimulate NB metastasis from a primary tumor site.

Discussion

It is believed that chemokines and chemokine receptors form a complicated network in tumor progression [20]. Both their expression profiles and regulatory mechanisms have yet to be completely elucidated. To establish the role of the chemokine system in NB metastasis, we first profiled five chemokines and receptors (CCL2, CCL3, SDF-1, CXCR4, and CCR7) and demonstrated differential expression in eight NB cell lines and one primary culture from an NB patient sample, NB12. These five chemokines and receptors have been reported frequently for their potential role in the metastasis of other tumor types to the bone marrow, liver, lymph node, bone, or skin [21–25]—all common metastatic sites in NB patients. Our studies demonstrated that CXCR4 is expressed in most NB cell lines and in the primary tumor sample NB12, which points to its potential involvement in NB metastasis. Consistent with our finding, CXCR4 expression was reported in other NB cell lines (including SH-SY5Y and SK-N-MC), and CXCR4 was proven to be functional because it could bind its ligand SDF-1 on the cell surface, inducing a rapid and transient intracellular calcium increase in SK-N-SH cells [26].

Our key findings showed that in NB cells, the chemokine/receptor system is a dynamic system that can be dynamically modulated. A similar phenomenon was observed in dendritic cell (DC) maturation where circulating DC precursors typically express just two chemokine receptors, CCR2 and CXCR4. On entering tissues and differentiating into immature DC, they express CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR2, and CXCR4. As these cells mature, expression of these receptors is gradually lost, whereas expression of a single lymphoid chemokine receptor CCR7 is rapidly induced [27,28]. Studies in NB and other cancer types indicate that different cell lines express different levels of chemokine receptors. Even for the same cell line, different independent studies demonstrated different levels [9,18]. All these findings and our results suggest that the expression of chemokine receptors could change in response to changes in their microenvironments, especially during different stages in NB metastasis.

Although it seems clear that some chemokine receptors, especially CXCR4, are often highly expressed in malignant tumors [9], the mechanism responsible for its upregulation has not been completely elucidated. Tumor–stromal interaction could be a potential mechanism. Tumor–stromal interaction is a very complicated process in tumor progression. Most studies on stromal cells have focused on integrins, growth factors, and cytokines [29–31]. In this study, we explored tumor–stromal interaction through the chemokine/receptor system and demonstrated for the first time that, in NB, different stromata have divergent effects on the expression of chemokine receptors. Another important finding in this study is that, in NB, the divergent effects of different stromata on chemokine receptor expression are achieved by the release of IL-5 and IFN-γ at differential levels by stromal cells, which indicates that tumor–stromal interaction could regulate the movement of NB cells to distant sites by modulating the chemokine/receptor system. It has been demonstrated that CXCR4 expression can be regulated positively by cytokines such as TGF-β1, VEGF, and bFGF, and can be regulated negatively by cytokines such as IL-5 and IFN-α in leukocytes, endothelial cells, and neural cells [32–36]. It was also demonstrated that hypoxia strongly affects the chemokine system in macrophages [37]. Human macrophages exposed to low-oxygen conditions do not migrate in response to CCL12 in hypoxic conditions [38]. By contrast, CXCR4 expression and function in myeloid populations are strongly increased through the activation of hypoxia-inducible factor-1α [39]. There are very limited published data on cytokine concentration in tissue microenvironments. Hogan and Landreth [40] detected the production of IL-5 in bone marrow stromal cells. It was also demonstrated that local overexpression of IFN-γ in stromal cells was much more potent than the exogenous addition of soluble cytokine to culture media [41]. Our *in vitro* studies demonstrated the regulation of CXCR4 expression by IL-5 and IFN-γ at the nanomolar level. It is therefore conceivable that physiological significant effects could be achieved in *in vivo* microenvironments.

Chemokines and their receptors constitute a key chemoattractant mechanism used by both normal and tumor cells [1–5]. So far, limited studies have been carried out on the role of CXCR4 in NB metastasis, and unexplained inconsistency was observed among results from different studies [18,19,42]. We suspected that this discrepancy could result

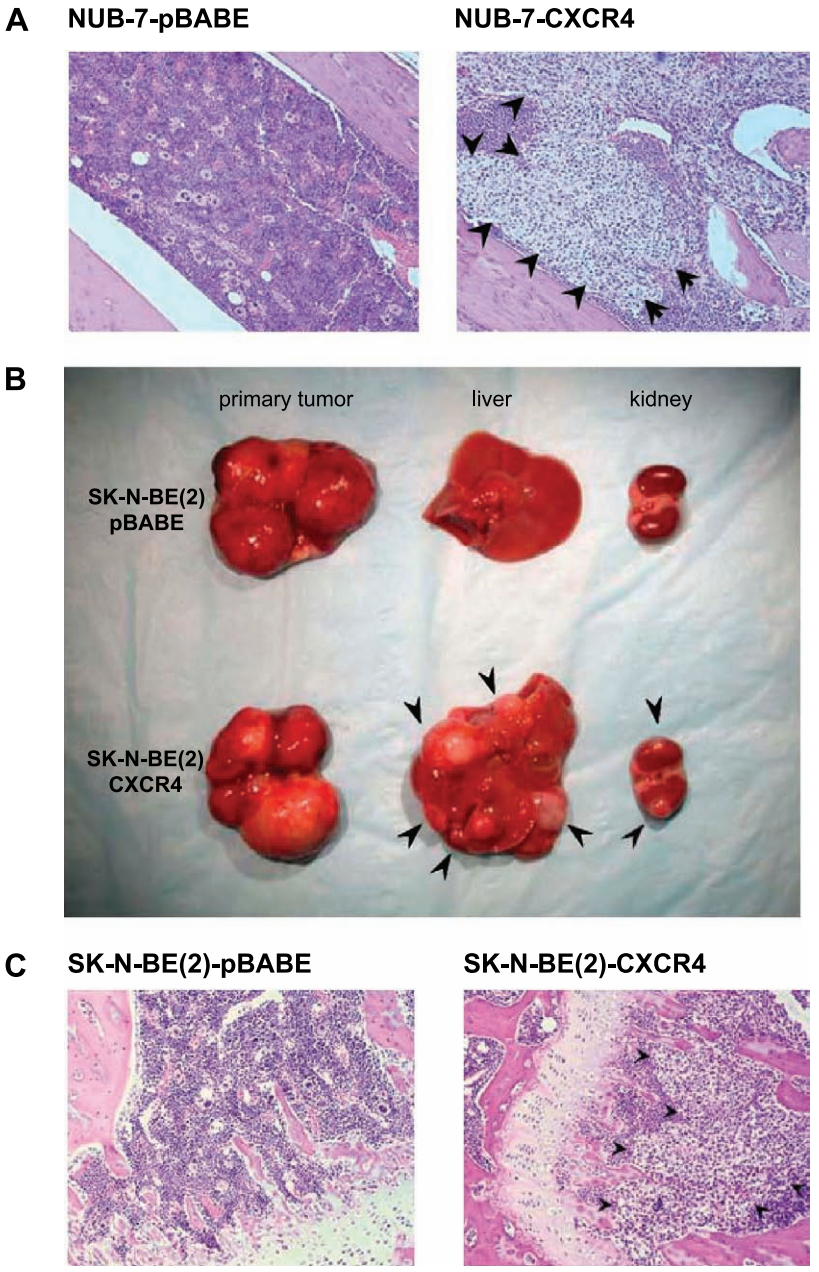


Figure 5. *CXCR4 expression regulates NB metastasis in a mouse xenograft model. (A) Both CXCR4-overexpressing (NUB-7–CXCR4) and control NUB-7 cells (NUB-7–pBABE) were injected intravenously to NOD/SCID mice at a dose of 1 × 10^6 cells. Mice were euthanized after 3 weeks. Femurs were decalcified, and processed tissue sections were stained with hematoxylin–eosin. Disseminated metastasis was observed in the liver, kidney, and lung (detailed in Table 2). Extensive bone marrow metastasis was observed after CXCR4 overexpression. Photomicrographs of representative bone marrow sections are shown (original magnification, ×200). (B) CXCR4-overexpressing SK-N-BE(2) cells (1 × 10^6; SK-N-BE(2)–CXCR4) and control SK-N-BE(2) cells (SK-N-BE(2)–PBABE) were injected subcutaneously, with four mice in each group. After sacrificing the mice at 3 weeks, images of primary tumor, liver metastasis, and kidney metastasis were taken under bright field. Figures shown are representative of tissues from two groups. Liver and kidney metastases could only be observed in the CXCR4-overexpressing group pointed by arrows. (C) In the subcutaneous model, the CXCR4-overexpressing group (SK-N-BE(2)–CXCR4) also showed bone marrow metastasis from femur sections (original magnification, ×200).*

from the dynamic change of CXCR4 expression under different culture environments and relatively low levels of CXCR4 expression in NB cell culture. To observe a significant effect of CXCR4 in NB development, the expression level of CXCR4 has to be upregulated. Because the bone marrow is a key producing site for the CXCR4 ligand SDF-1, it is reasonable to propose that the release of SDF-1 would attract NB cells to form a secondary tumor at this site. Clinically, the most common location of NB metastases is the bone marrow (70%), but limited bone marrow metastasis can be achieved in current animal models, even with a highly malignant cell line derived from a bone marrow metastatic site [43]. We suspected that this altered metastatic pattern in animal models could result from a reduced or an even totally silenced expression of certain chemokine receptor(s) in NB cell lines due to the change in microenvironment. Indeed, from

the chemokine profile shown in Figure 1*A*, the multirelapse patient sample NB12 has much higher levels of CXCR4, CCR7, and CCL3 compared to all NB cell lines.

To investigate this possibility, we overexpressed CXCR4 in the NB cell lines NUB-7 and SK-N-BE(2), and found that overexpression of CXCR4 promoted NB cell migration selectively toward bone marrow SCM *in vitro*. In a mouse xenograft model, we further demonstrated that bone marrow metastasis could be achieved successfully by CXCR4-overexpressed NB cells. These data collectively indicate that targeted bone marrow metastasis could be enhanced by the overexpression of CXCR4. We believe that the expression of CXCR4 could regulate the homing process part of metastatic cascade. In addition, CXCR4 expression could also be involved in some other steps for NB metastasis. Because different NB cells have various potentials to form metastasis in a subcutaneous xenograft model, we used SK-N-BE(2) cells in our subcutaneous model. Unexpectedly, we detected disseminated metastasis in addition to bone marrow metastasis from subcutaneously growing CXCR4-overexpressing SK-N-BE(2) cells. Taken together with some recent studies suggesting the regulation of angiogenesis by CXCR4 [44], we suggest that the dissemination of CXCR4-overexpressed SK-N-BE(2) cells from primary tumors could result from increased angiogenesis or local invasiveness.

Our studies make it apparent that CXCR4 expression can be upregulated by liver stromal cells, and this upregulation could stimulate NB metastasis toward the bone marrow, which then suggests that tissue modulation of chemokine receptors could be a critical step before NB metastasis. Our data also showed that adrenal SCM dramatically suppressed CXCR4 expression in NUB-7 cells. We propose that primary NB cells may have a limited expression of CXCR4 while residing in the adrenal gland. Only after the normal adrenal gland had been compromised by tumor growth could CXCR4 expression recover and thereby facilitate tumor cell migration toward other SDF-1–expressing organs, such as the bone marrow. Consequently, after the bone marrow had been replaced, tumor cells could again migrate to another metastatic site. Thus, NB tumor cells might be chemoattracted to new sites as older ones become less conducive to supporting tumor growth—a process governed potentially by chemokine/receptor activity. Taken together, we suggest that, to stimulate tumor cell migration toward favorable metastatic sites, the expression of the chemokine system has to be modulated accordingly. To sum up, our observations give credence to the earlier ''seed-and-soil'' hypothesis [45] and invoke the chemokine/receptor system as a dynamic system that is modulated in different microenvironments and targets NB cells to different organs according to specific chemokine/receptor expression profiles.

In addition to CXCR4 and SDF-1, other chemokine and cognate receptors could also take part in NB metastasis. The expression of CCR7 and CCR10 in melanoma cells was linked to the expression of cognate ligands at the two major sites of metastasis: skin and lymph nodes [46]. Another chemokine LARC/CCL20 is constitutively expressed in the liver, and this ligand interacts with CCR6. The latter receptor is commonly overexpressed in colon, thyroid, and ovarian carcinomas, which may contribute to the commonly observed metastases of these tumors to the liver [47]. In additional studies, we also found the expression of CCR6 in some NB cell lines (unpublished observations), which may explain why the liver is one of the most frequent metastatic sites in both patients and xenograft models. Interestingly, the primary clinical sample NB12 expressed all three receptors, including CXCR4, CCR7, and CCR6, suggesting perhaps a greater degree of heterogeneity in comparison to cell lines that are more phenotypically homogeneous. Whether primary NB consists of tumor cells with multiple metastatic phenotypes is an important question and has many ramifications. Further studies are needed to address the link between chemokine/receptor profile and clinical progression.

In conclusion, the chemokine/receptor profile generated for NB in the present study provides a definite link between NB metastasis and expression of the chemokine/receptor system. In addition, by modulating chemokine receptor expression, we provide a novel means for manipulating and enhancing NB tumor cell invasiveness and metastasis and, thereby, the means to develop site-directed NB metastasis models. Because such models would reflect NB progression in patients, they could mimic tumor behavior more accurately and would constitute better models to evaluate new therapeutics. Clinically, the demonstration of a central role for the chemokine/receptor system in NB metastasis also provides an alternative target for NB therapy by blocking chemokine/receptor function. In addition, if it were possible to demonstrate a relationship between chemokine (or receptor) expression profiles and different stages of NB, this could lead to alternative prognostic makers for NB and a strategy to enhance both diagnostic and therapeutic modalities.

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References

[1] Charo IF and Ransohoff RM (2006). The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* **354,** 610–621.

[2] Laudanna C and Alon R (2006). Right on the spot. Chemokine triggering of integrin-mediated arrest of rolling leukocytes. *Thromb Haemost* **95,** 5–11.

[3] Bajetto A, Bonavia R, Barbero S, Florio T, and Schettini G (2001). Chemokines and their receptors in the central nervous system. *Front Neuroendocrinol* **22,** 147–184.

[4] Balkwill F and Mantovani A (2001). Inflammation and cancer: back to Virchow? *Lancet* **357,** 539–545.

[5] Vicari AP and Caux C (2002). Chemokines in cancer. *Cytokine Growth Factor Rev* **13,** 143–154.

[6] Ward SG, Bacon K, and Westwick J (1998). Chemokines and T lymphocytes: more than an attraction. *Immunity* **9,** 1–11.
[7] Kuang Y, Wu Y, Jiang H, and Wu D (1996). Selective G protein coupling by C–C chemokine receptors. *J Biol Chem* **271,** 3975–3978.
[8] Sozzani S, Molino M, Locati M, Luini W, Cerletti C, Vecchi A, and Mantovani A (1993). Receptor-activated calcium influx in human monocytes exposed to monocyte chemotactic protein-1 and related cytokines. *J Immunol* **150,** 1544–1553.
[9] Balkwill F (2004). The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin Cancer Biol* **14,** 171–179.
[10] Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, and McCauley LK (2002). Use of the stromal cell–derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* **62,** 1832–1837.
[11] Scotton CJ, Wilson JL, Scott K, Stamp G, Wilbanks GD, Fricker S, Bridger G, and Balkwill FR (2002). Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. *Cancer Res* **62,** 5930–5938.
[12] Rempel SA, Dudas S, Ge S, and Gutierrez JA (2000). Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. *Clin Cancer Res* **6,** 102–111.
[13] Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410,** 50–56.
[14] Koshiba T, Hosotani R, Miyamoto Y, Ida J, Tsuji S, Nakajima S, Kawaguchi M, Kobayashi H, Doi R, Hori T, et al. (2000). Expression of stromal cell–derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. *Clin Cancer Res* **6,** 3530–3535.
[15] Burger M, Glodek A, Hartmann T, Schmitt-Graff A, Silberstein LE, Fujii N, Kipps TJ, and Burger JA (2003). Functional expression of CXCR4 (CD184) on small-cell lung cancer cells mediates migration, integrin activation, and adhesion to stromal cells. *Oncogene* **22,** 8093–8101.
[16] Kryczek I, Wei S, Keller E, Liu R, and Zou W (2006). Stromal derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *Am J Physiol Cell Physiol* (Epub ahead of print).
[17] Wysoczynski M, Reca R, Ratajczak J, Kucia M, Shirvaikar N, Honczarenko M, Mills M, Wanzeck J, Janowska-Wieczorek A, and Ratajczak MZ (2005). Incorporation of CXCR4 into membrane lipid rafts primes homing-related responses of hematopoietic stem/progenitor cells to an SDF-1 gradient. *Blood* **105,** 40–48.
[18] Geminder H, Sagi-Assif O, Goldberg L, Meshel T, Rechavi G, Witz IP, and Ben Baruch A (2001). A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell–derived factor-1, in the development of bone marrow metastases in neuroblastoma. *J Immunol* **167,** 4747–4757.
[19] Russell HV, Hicks J, Okcu MF, and Nuchtern JG (2004). CXCR4 expression in neuroblastoma primary tumors is associated with clinical presentation of bone and bone marrow metastases. *J Pediatr Surg* **39,** 1506–1511.
[20] Balkwill F (2004). Cancer and the chemokine network. *Nat Rev Cancer* **4,** 540–550.
[21] Ghadjar P, Coupland SE, Na IK, Noutsias M, Letsch A, Stroux A, Bauer S, Buhr HJ, Thiel E, Scheibenbogen C, et al. (2006). Chemokine receptor CCR6 expression level and liver metastases in colorectal cancer. *J Clin Oncol* **24,** 1910–1916.
[22] Menten P, Saccani A, Dillen C, Wuyts A, Struyf S, Proost P, Mantovani A, Wang JM, and Van Damme J (2002). Role of the autocrine chemokines MIP-1alpha and MIP-1beta in the metastatic behavior of murine T cell lymphoma. *J Leukoc Biol* **72,** 780–789.
[23] Lebrecht A, Grimm C, Lantzsch T, Ludwig E, Hefler L, Ulbrich E, and Koelbl H (2004). Monocyte chemoattractant protein-1 serum levels in patients with breast cancer. *Tumour Biol* **25,** 14–17.
[24] Murakami T, Cardones AR, and Hwang ST (2004). Chemokine receptors and melanoma metastasis. *J Dermatol Sci* **36,** 71–78.
[25] Mashino K, Sadanaga N, Yamaguchi H, Tanaka F, Ohta M, Shibuta K, Inoue H, and Mori M (2002). Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res* **62,** 2937–2941.
[26] Banisadr G, Dicou E, Berbar T, Rostene W, Lombet A, and Haour F (2000). Characterization and visualization of [^{125}I] stromal cell–derived factor-1alpha binding to CXCR4 receptors in rat brain and human neuroblastoma cells. *J Neuroimmunol* **110,** 151–160.
[27] Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, Qin S, and Lanzavecchia A (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* **28,** 2760–2769.
[28] Sozzani S, Luini W, Borsatti A, Polentarutti N, Zhou D, Piemonti L, D'Amico G, Power CA, Wells TN, Gobbi M, et al. (1997). Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J Immunol* **159,** 1993–2000.
[29] Park CC, Bissell MJ, and Barcellos-Hoff MH (2000). The influence of the microenvironment on the malignant phenotype. *Mol Med Today* **6,** 324–329.
[30] Bernards R (2003). Cancer: cues for migration. *Nature* **425,** 247–248.
[31] Varani J (1987). Interaction of tumor cells with the extracellular matrix. *Revis Biol Cell* **12,** 1–113.
[32] Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ, and Oppenheim JJ (1999). Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: *in vivo* neovascularization induced by stromal-derived factor-1alpha. *Am J Pathol* **154,** 1125–1135.
[33] Rostasy K, Gorgun G, Kleyner Y, Garcia A, Kramer M, Melanson SM, Mathys JM, Yiannoutsos C, Skolnik PR, and Navia BA (2005). Tumor necrosis factor alpha leads to increased cell surface expression of CXCR4 in SK-N-MC cells. *J Neurovirol* **11,** 247–255.
[34] Franitza S, Kollet O, Brill A, Vaday GG, Petit I, Lapidot T, Alon R, and Lider O (2002). TGF-beta1 enhances SDF-1alpha–induced chemotaxis and homing of naive T cells by up-regulating CXCR4 expression and downstream cytoskeletal effector molecules. *Eur J Immunol* **32,** 193–202.
[35] Iikura M, Miyamasu M, Yamaguchi M, Kawasaki H, Matsushima K, Kitaura M, Morita Y, Yoshie O, Yamamoto K, and Hirai K (2001). Chemokine receptors in human basophils: inducible expression of functional CXCR4. *J Leukoc Biol* **70,** 113–120.
[36] Shirazi Y and Pitha PM (1998). Interferon downregulates CXCR4 (*fusin*) gene expression in peripheral blood mononuclear cells. *J Hum Virol* **1,** 69–76.
[37] Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, Bernasconi S, Saccani S, Nebuloni M, Vago L, et al. (2003). Regulation of the chemokine receptor CXCR4 by hypoxia. *J Exp Med* **198,** 1391–1402.
[38] Turner L, Scotton C, Negus R, and Balkwill F (1999). Hypoxia inhibits macrophage migration. *Eur J Immunol* **29,** 2280–2287.
[39] Ceradini DJ and Gurtner GC (2005). Homing to hypoxia: HIF-1 as a mediator of progenitor cell recruitment to injured tissue. *Trends Cardiovasc Med* **15,** 57–63.
[40] Hogan MB and Landreth KS (2000). IL-5 production by bone marrow stromal cells: implications for eosinophilia associated with asthma. *J Allergy Clin Immunol* **106,** 329–336.
[41] Selleri C, Maciejewski JP, Sato T, and Young NS (1996). Interferon-gamma constitutively expressed in the stromal microenvironment of human marrow cultures mediates potent hematopoietic inhibition. *Blood* **87,** 4149–4157.
[42] Airoldi I, Raffaghello L, Piovan E, Cocco C, Carlini B, Amadori A, Corrias MV, and Pistoia V (2006). CXCL12 does not attract CXCR4$^+$ human metastatic neuroblastoma cells: clinical implications. *Clin Cancer Res* **12,** 77–82.
[43] Thompson J, Guichard SM, Cheshire PJ, Richmond LB, Poquette CA, Ragsdale ST, Webber B, Lorsbach R, Danks MK, and Houghton PJ (2001). Development, characterization and therapy of a disseminated model of childhood neuroblastoma in SCID mice. *Cancer Chemother Pharmacol* **47,** 211–221.
[44] Chen GS, Yu HS, Lan CCE, Chow KC, Lin TY, Kok LF, Lu MP, Liu CH, and Wu MT (2006). CXC chemokine receptor CXCR4 expression enhances tumorigenesis and angiogenesis of basal cell carcinoma. *Br J Dermatol* **154,** 910–918.
[45] Paget S (1889). The distribution of secondary growths in cancer of the breast. *Lancet* **133,** 571–573.
[46] Payne AS and Cornelius LA (2002). The role of chemokines in melanoma tumor growth and metastasis. *J Invest Dermatol* **118,** 915–922.
[47] Schutyser E, Struyf S, and Van Damme J (2003). The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* **14,** 409–426.